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# Cloning and Characterization of the Canine Photoreceptor Specific Cone-Rod Homeobox (CRX) Gene and Evaluation as a Candidate for Early Onset Photoreceptor Diseases in the Dog

## Abstract

### PURPOSE:

The cone-rod homeobox protein (CRX) is a member of the homeodomain-containing protein family expressed in the retinal photoreceptors and pinealocytes; it is involved in the regulation of the coordinate expression of multiple photoreceptor specific genes during retinal development. Mutations in the CRX gene are causally associated with retinal degeneration phenotypes in man. To clone the full length cDNA, characterize the genomic organization of canine CRX, map the gene in a radiation hybrid (RH) panel, and evaluate it as a candidate for canine inherited retinal degenerations.

### METHODS:

cDNA representational difference analysis (RDA) was done using normal and cone degeneration (cd) affected retinas. Exonic primers designed from consensus sequences of mammalian CRX cDNA were used to amplify and sequence dog genomic DNA. Canine specific primers were used for RH mapping of CRX on the RH3000 cell line. Linkage, sequencing and/or mapping the disease locus was used to evaluate CRX as a disease associated candidate gene.

### RESULTS:

The gene comprises three exons and two introns and codes for a transcript with a 900 bp open reading frame (ORF). In agreement with human map data, RH mapping placed canine CRX on the proximal end of CFA1, in a region of synteny with HSA19q13-q13.3. Based on RH mapping, meiotic linkage or sequencing data, we excluded CRX as the cause of canine early onset photoreceptor degenerations affecting Alaskan malamutes (cd), collies (rod-cone dysplasia 2, rcd2), American Staffordshire terriers, and Tibetan terriers.

### CONCLUSIONS:

Canine CRX has a high level of nucleotide and amino acid sequence identity with orthologous sequences reported for other species. The gene is excluded from causal association with 4 early onset photoreceptor diseases affecting cones (cd) or rods and cones (rcd2, PRA in American Staffordshire terriers, and Tibetan terriers).

## Disciplines

Comparative and Laboratory Animal Medicine | Eye Diseases | Medicine and Health Sciences | Ophthalmology | Veterinary Medicine

## Comments

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# Cloning and characterization of the canine photoreceptor specific cone-rod homeobox (CRX) gene and evaluation as a candidate for early onset photoreceptor diseases in the dog

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**Purpose:** The cone-rod homeobox protein (CRX) is a member of the homeodomain-containing protein family expressed in the retinal photoreceptors and pinealocytes; it is involved in the regulation of the coordinate expression of multiple photoreceptor specific genes during retinal development. Mutations in the CRX gene are causally associated with retinal degeneration phenotypes in man. To clone the full length cDNA, characterize the genomic organization of canine CRX, map the gene in a radiation hybrid (RH) panel, and evaluate it as a candidate for canine inherited retinal degenerations.

**Methods:** cDNA representational difference analysis (RDA) was done using normal and cone degeneration (cd) affected retinas. Exonic primers designed from consensus sequences of mammalian CRX cDNA were used to amplify and sequence dog genomic DNA. Canine specific primers were used for RH mapping of CRX on the RH3000 cell line. Linkage, sequencing and/or mapping the disease locus was used to evaluate CRX as a disease associated candidate gene.

**Results:** The gene comprises three exons and two introns and codes for a transcript with a 900 bp open reading frame (ORF). In agreement with human map data, RH mapping placed canine CRX on the proximal end of CFA1, in a region of synteny with HSA19q13-q13.3. Based on RH mapping, meiotic linkage or sequencing data, we excluded CRX as the cause of canine early onset photoreceptor degenerations affecting Alaskan malamutes (cd), collies (rod-cone dysplasia 2, rcd2), American Staffordshire terriers, and Tibetan terriers.

**Conclusions:** Canine CRX has a high level of nucleotide and amino acid sequence identity with orthologous sequences reported for other species. The gene is excluded from causal association with 4 early onset photoreceptor diseases affecting cones (cd) or rods and cones (rcd2, PRA in American Staffordshire terriers, and Tibetan terriers).

The cone-rod homeobox protein (CRX) is a member of the homeodomain-containing protein family, and is expressed in retinal photoreceptors and pinealocytes [1,2]. As a transcription factor, CRX is involved in vertebrate eye development and plays an important role in gene expression. CRX binds to conserved sites of several photoreceptor specific genes in the retina, and transactivates their expression during photoreceptor differentiation and maturation. Microarray analysis of retinas from wild type and CRX-deficient mice has indicated that the expression of 10 out of 17 differentially expressed genes controlled by CRX are photoreceptor specific or photoreceptor enriched. All of these target genes contain consensus CRX-binding elements [3]. Furthermore, both the leucine zipper domain of NRL (neural retinal leucine zipper) and the DBD2 and DBD3 domains of HMG I (Y) (non-histone chromosomal proteins high mobility group I (Y)) have been observed to act synergistically with CRX in the enhancement of expression

of some photoreceptor specific genes. These interactions are crucial, for example, in the regulation of rhodopsin expression [4,5].

Because of its critical role in the early stages of photoreceptor development, CRX is an excellent candidate gene for early onset retinal diseases [1]. This has been confirmed by the identification of mutations that result in cone-rod dystrophy [6,7] and Lebers congenital amaurosis [8-10]. In addition, a wide range of clinical retinal phenotypes have been associated with mutations in this gene [11-15].

The impetus for the present study arose from an ongoing project to identify and enable evaluation of candidate genes for the large number of inherited canine photoreceptor disorders for which causes have not yet been identified. To this end we identified canine CRX (cCRX) as a specifically attractive candidate for cloning and evaluation. In parallel, we undertook to identify other potential candidate genes using Representational Difference Analysis (RDA). RDA was used to find genes expressed differentially between normal canine retinas and retinas affected with cone degeneration (cd). One of the clones identified by RDA turned out to be the cCRX cDNA. We thus report here the cloning of cCRX by two different but

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complementary strategies, the characterization of its genomic organization, and its exclusion as the cause of canine cd in Alaskan malamutes, rod cone dysplasia type 2 (rcd2) in collies, and two different forms of early onset progressive retinal atrophy (PRA) affecting the American Staffordshire terrier and the Tibetan terrier, respectively.

## METHODS

**Animals, pedigrees and samples:** Blood samples were obtained from privately owned Tibetan terrier dogs affected with an autosomal recessive form of progressive retinal atrophy (PRA). This form of PRA is clinically evident by 1-1.5 years and progresses to complete blindness at a young age [16]. Blood and tissue samples from all other dogs studied were obtained from the Retinal Disease Studies Facility (RDSF) colony maintained under the sponsorship of NEI/NIH grant EY-06855 (see below). Genomic DNA was isolated from citrated blood samples either by standard phenol/chloroform extraction, or a rapid protocol described previously [17].

To enable identification of potential retinal degeneration genes by RDA, normal canine retinas and retinas from cd affected dogs [18-20] were obtained from the RDSF colony. Blood, retinal and other tissue samples were also obtained from 2 other strains of dogs from the RDSF colony. Each such strain represented a different canine pedigree segregating a phenotypically distinct retinal disease. The rcd2 strain segregates an autosomal recessive rod-cone defect originally derived from collie dogs [21,22]. The other strain segregates a different autosomal recessive early onset retinal degeneration derived from the American Staffordshire terrier breed. The latter disease is as yet incompletely characterized, but affected animals have severe visual impairment by 6 months of age which progresses rapidly to total blindness (Acland and Aguirre, unpublished). All procedures involving animals were done in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Isolation of RNA, cDNA synthesis, and cDNA Representational Difference Analysis (RDA):** Total RNA was isolated from normal and cd affected canine retinas, both samples from animals 2 years of age, using RNAzol B (Tel-Test Inc., Friendswood, TX). At 2 years of age, the cd affected retinas would have nearly complete loss of cones, thus enriching for cone transcripts that would be expressed in the normal retina. Poly(A)<sup>+</sup> RNAs were fractionated from total RNA by two

successive rounds on oligo(dT)-cellulose columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ). First strand cDNA synthesis was performed with Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA) using 2 µg of mRNA and 1 µg of oligo(dT) primer. Then the first strand cDNA was used as a template to create the second strand cDNA, as described in the manufacturer's protocol (Life Technologies Inc., Gaithersburg, MD).

Generation of fragments for RDA was carried out according to the protocol of Hubank and Schatz [23], and based on genomic representational difference analysis [24]. Double stranded cDNA (4 µg) was digested with DpnII and amplified cDNA fragments (amplicons) were obtained using different sets of primers: R-Bgl-12 (GAT CTG CGG TGA), R-Bgl-24 (AGC ACT CTC CAG CCT CTC), J-Bgl-12 (GAT CTG TTC ATG), J-Bgl-24 (ACC GAC GTC GAC TAT CCA TGAACA), N-Bgl-12 (GAT CTT CCC TCG), and N-Bgl-24 (AGG CAA CTG TGC TAT CCG AGG GAA). Three rounds of subtractive hybridization were performed and the RDA products were digested with DpnII and ligated to pBluescript KS II<sup>+</sup> (Stratagene, La Jolla, CA). Sequence of the cDNA inserts was determined using the Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

**Genomic and cDNA Cloning:** In order to obtain complete genomic sequence coverage of cCRX, forward consensus primer crx12 from exon I was used in combination with

**TABLE 1. PRIMERS USED FOR CLONING CANINE CRX AND FOR RADIATION HYBRID MAPPING**

Sequence	Primer ID	Primer Location
CAGCTTGGTGCATCAGATCCACG	crx-3	Exon I R' primer
GGTGGCTCTGAAGATCAACCTACC	crx-6	Exon II F' primer
GCAGGACGAGCCTTGGTCTGTGC	crx-7	Exon III R' primer
CTACAAGATCTGAAACTTCCAGG	crx-8	Exon III R' primer
CATGATGGCGTATATGAACCCG	crx-12	Exon I F' primer
TTGCCAGCCACACTGCTGG	crx-13	Intron II F' primer
TCCGTCTAGGTCTCATTGAAGG	crx-14	Intron II R' primer
CAGTCCACGGATGTAACCCC	crx-15	Intron II F' primer
GCTCAACCGTTGAGCCATCCAG	crx-16	Intron I F' primer
GGGGTTACATCCGTGGAGCTG	crx-18	Intron II R' primer
GGATGGCTCAACGTTGAGCATC	crx-19	Intron I R' primer
CGGCACGAGGACTCTGACTC	crx-20	5'UTR primer
GGAACCATGGAGGATACTTCGGG	crx-21	Intron II F' primer
TACCGAGCTCGGATCCACTA	pCRII-1	Vector primer
TTAAGCGGGATATCACTCAGC	T7	Vector primer

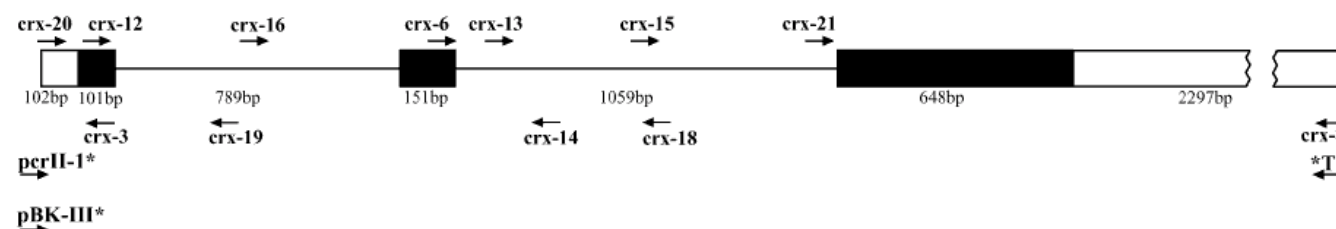


Figure 1. Genomic organization of cCRX. The location of gene specific and vector (\*) primers used in cDNA and genomic cloning and characterization is indicated. The location of other CRX primers included in Table 1 are also indicated. Boxes identify exons (open denote 5' and 3' UTR, shaded ORF); lines indicate introns.



consensus reverse primer crx-8 from exon III in a PCR reaction on 100 ng of genomic DNA. (Figure 1). The resulting PCR product was purified using the QiaexII gel extraction kit (Qiagen, Chatsworth, CA), and cloned using the TA Cloning kit (Invitrogen, San Diego, CA). Vector primers T7 and pcrII-1 were used to obtain the 5' and 3' sequences of the genomic clone. Exonic primers designed from consensus sequences were used to sequence the CRX clone in order to establish intron sizes; intronic primers were designed from the canine clone sequence to give full genomic sequence coverage of CRX. In order to obtain 5'UTR sequence, exon I reverse primer crx-3 was used with vector primer pBK-III in a PCR reaction with DNA extracted from a custom canine retinal cDNA library (Stratagene, La Jolla, CA). The resulting PCR product was cloned (as above) and sequenced using vector specific primers. Sequencing in an Applied Biosystems ABI 377 automated DNA sequencer at the core sequencing facility of Cornell University verified the authenticity of the CRX clones. Table 1 gives details on primer sequences and PCR conditions used for genomic cloning of cCRX.

**Radiation hybrid (RH) mapping:** Exonic consensus primers were designed from CRX cDNA sequences available in

GenBank for mouse, rat, bovine, and human. A forward primer (crx-6) from exon II was used in a canine genomic PCR reaction with a reverse primer (crx-7) designed from exon III. The resulting PCR product of 1200 nucleotides was purified using the QiaexII gel extraction kit, eluted in water, and sequenced using the crx-6 primer in the sequencing reaction. From the resulting sequence, two intronic primers from intron II, crx-13 and crx-14, were designed to produce a canine specific PCR product of 174 base pairs (Table 1). The crx-13 and crx-14 primers were tested on canine, hamster, and 5:1 hamster/canine DNA under universal conditions (94 °C (20 s), 58 °C (20 s), 74 °C (20 s); 30 cycles, 2.0 mM Mg<sup>2+</sup>, DNA 50 ng/μl) [25] for use on a whole genome dog/hamster RH3000 panel (92 RH cell RH083000, Research Genetics, Inc., Huntsville, AL). To establish linkage to CFA1, we amplified five markers (VASP, FH2634, C5RI, LHB and FH2326) previously published for the region of interest [26]. In addition we used primers FH3505-f (5' ACTGGCCAAAGAGTACAAGG 3') and FH3505-r (5' AGTCATGCTGATTTCTGTGC 3') to add marker FH3505 to the canine RH map. The map was constructed using Multimap software [27] based on best two point analysis, placing markers at Lod score 2.0 for overall order.

## RESULTS

**Isolation and characterization of cCRX cDNA by RDA and library screening:** The different cDNA fragments obtained from RDA/PCR amplification of mRNAs from normal and cd dog retinas were subcloned into the pCR II cloning vector (Invitrogen). Through sequence analysis and Genbank database search, one of these fragments was found to have a high degree of identity to the published human and mouse CRX cDNA. This fragment was subsequently used to screen approximately 100,000 plaques from a custom made canine retinal cDNA library (Stratagene). Two positive clones were identified, containing insert sizes of 2.2 and 3.4 kb. The longer insert contained the entire sequence of the canine CRX cDNA with an unusually long 3' untranslated region (UTR) of 2297 bp, 102 bp of the 5' UTR, as well as the open reading frame of 900 bp encoding a protein of 299 amino acids. This sequence is similar to that obtained by cDNA library screening and cloning. The complete cCRX cDNA sequence was compiled by overlaying sequence data from the two different strategies used, and the contig represented consensus sequence information (GenBank accession number AF454668). Alignment of amino acid sequences of cCRX with those of bovine, human, rat and mouse showed a high degree of homology between these species with complete conservation of the homeodomain (Figure 2).

**Characterization of genomic cCRX:** Determination of the genomic organization of the canine CRX gene was done by PCR with primers designed from the canine sequence (Table 1). The positions of the exon and intron splice junctions, and the primers used are shown in Figure 1. Comparative analysis of the cCRX genomic and cDNA sequences revealed that the gene structure consists of three exons and two introns. The 5'-UTR and protein initiation codon as well as amino acids 2-34 are found in exon 1. Exon 2 contains the cDNA sequence en-

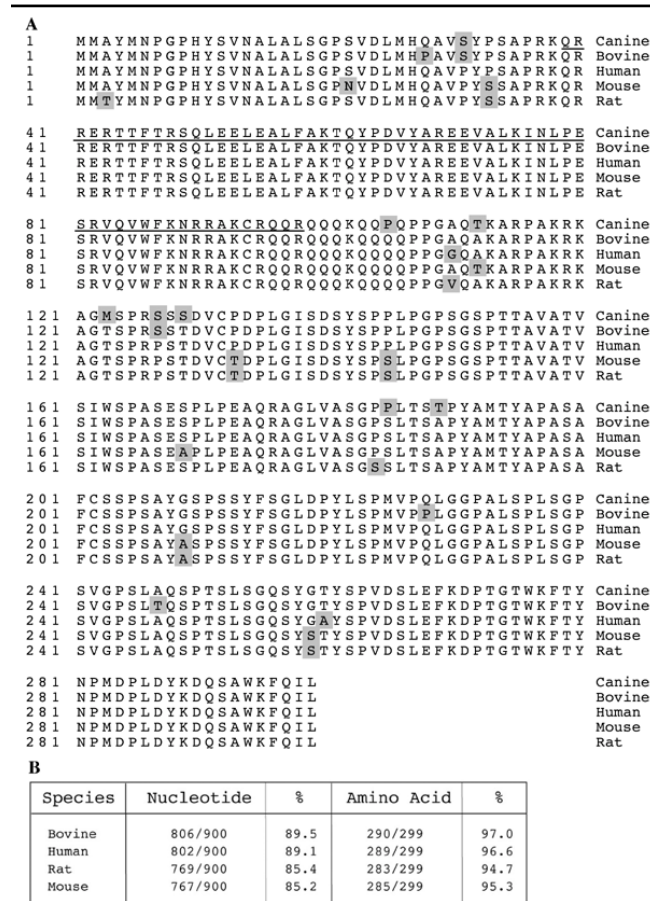


Figure 2. CRX sequence comparisons. **A:** The deduced amino acid sequences of canine CRX and comparison with other species; the conserved homeodomain is underlined. **B:** Canine CRX shows a high degree of homology at the nucleotide and amino acid levels with other species.

coding 50 aa residues from 35 to 84. Exon 3 spans the remaining 215 aa, and contains the entire 3'-UTR sequence. The sizes of the two introns are 789 and 1059 bp, respectively.

**Radiation hybrid mapping:** RH mapping using the RH083000 canine-hamster panel placed cCRX on the telomeric end of CFA1, in a position approximately 113.6 cR3000 telomeric to the tetranucleotide microsatellite FH2326, and 31.7 cR3000 centromeric to the tetranucleotide microsatellite FH2634 (Figure 3). Even though CRX and marker FH3505 have not been previously mapped in the dog, our RH3000 map is in complete agreement with the most recent publication of the CNRS RHDF5000 canine radiation hybrid map [28]. This region exhibits conserved synteny with HSA19q13-q13.3, and is thus in accord with human mapping data which localizes CRX to HSA19q13.

**Evaluation of cCRX as a candidate for inherited photoreceptor degeneration:** *Cone degeneration in the Alaskan malamute:* The mapping of cCRX to CFA1 excluded this gene as a candidate for cd. In other studies (Acland et al., unpublished), we had previously observed multiple recombinations between cd and markers FH2309, AHT138, C01.643, C01.164, FH2326, and FH2294 which have been mapped to CFA 1 [28]. This effectively excludes the entire interval flanking cCRX from association with the disease. In addition, northern blot hybridization of a CRX cDNA probe to canine retinal RNA from normal (16 weeks of age) and cd affected (6 weeks of age) animals showed no detectable transcriptional differences (data not shown).

*rcd2 in the collie:* The sequence of the normal canine CRX was compared to that of the rcd2 affected dogs. Full length genomic clones and overlapping PCR products were

sequenced in both directions to provide full genomic coverage of the gene. No differences in the sequence were observed between the normal and affected. Linkage analysis in an rcd2 pedigree informative for FH2634 demonstrated 2 recombinants among 4 informative dogs in a phase known pedigree (Figure 4A). The normal genomic sequencing and identification of 2 of 4 recombinations in a marker physically proximate to CRX effectively rules out the gene from causal association with the disease.

*Early onset retinal degeneration in the American Staffordshire terrier:* Genomic analysis indicated no sequence differences between normal and affected American Staffordshire terriers. Microsatellites FH2326 and FH2634 were informative for linkage analysis in the pedigree. These markers flank CRX and are tightly linked to each other with no recombina-

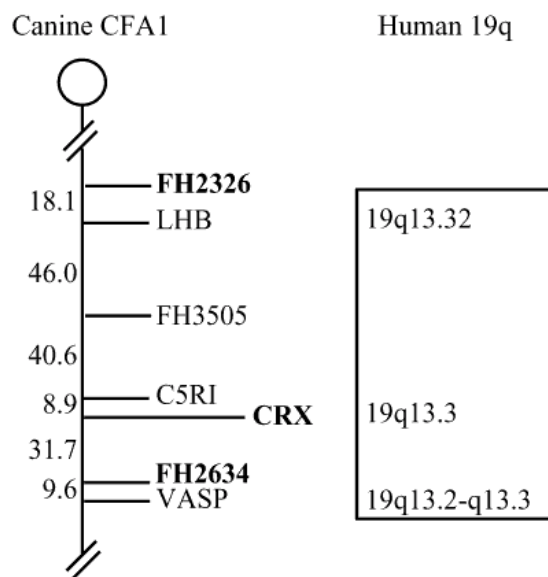


Figure 3. Radiation hybrid mapping of cCRX on the whole genome dog/hamster RH3000 panel. The gene is mapped to the centromeric part of CFA1 in a region of conserved synteny with HSA19q13-q13.3 (human cytogenetic position of gene markers is indicated in the right panel). Tetranucleotide markers FH2326 and FH2634 used for linkage analysis are shown in bold.

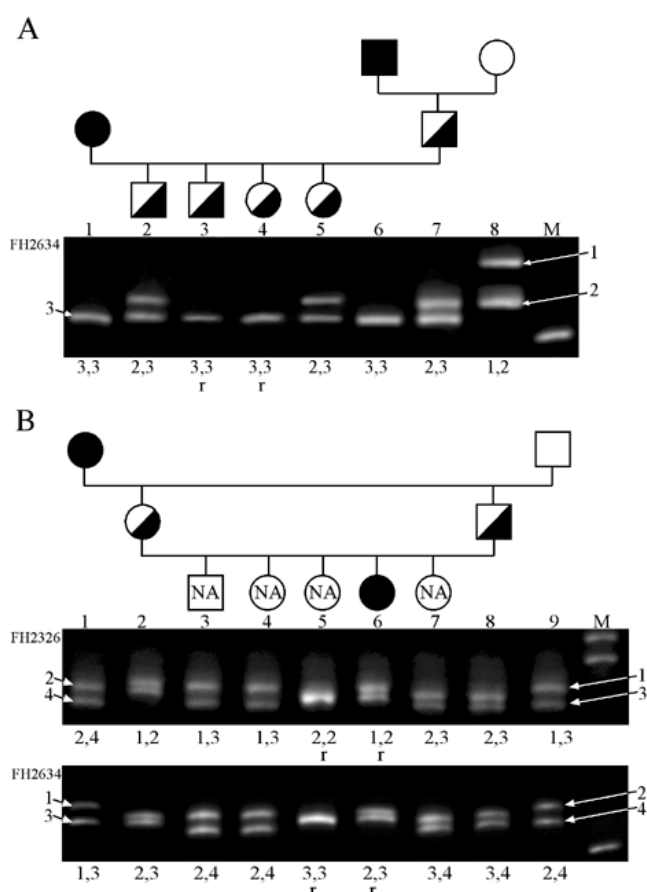


Figure 4. Linkage analysis in disease pedigrees. **A:** Linkage of FH2634 in an rcd2 informative pedigree. The different alleles are shown below the gel for each, and the two recombinant animals (r) identified. **B:** Linkage analysis of microsatellites FH2326 and FH2634 in an American Staffordshire terrier pedigree informative for early onset retinal degeneration. The different alleles are shown below the gel for each, and the recombinant animals (r) identified. The same animals (5, 6) are recombinant with both markers. Genotype of the pedigrees: square=male; circle=female; open, half shaded, shaded=normal, asymptomatic carrier, affected, respectively. NA=non-affected animal with heterozygous or homozygous normal genotype.

tions. Identifying 2 recombinant animals in the small subset pedigree effectively excludes CRX as the disease causing gene (Figure 4B).

**PRA in the Tibetan terrier:** The small number of animals available for study did not constitute a pedigree that could be used to examine for co-segregation between a polymorphism within markers flanking CRX and the disease. As an alternative strategy, we used genomic sequencing of the cCRX gene from a PRA affected Tibetan terrier, and compared the sequence obtained with that of normal. No differences were found, indicating the presumptive exclusion of this gene.

## DISCUSSION

In this study, we examined cCRX as a potential disease causing gene for inherited retinal disorders in the dog. Initially, we isolated and characterized the cCRX cDNA, determined its genomic structure and compared it with published mammalian orthologs (GenBank accession numbers: AF154123, AF024711, AF135838, NM\_007770). Sequence comparison revealed that the most divergent region of the CRX cDNAs is in the 3'-UTR which is unusually long, and has several cytidine rich (C-rich) segments.

Because CRX plays a critical role in early photoreceptor development and differentiation, we evaluated it as a candidate gene in a set of canine early onset diseases. These affect the rods and/or cones, and are phenotypically comparable to those which occur in man and result from mutations in CRX [1,6-10]. As part of a specific effort to identify potential candidates for canine cd, we isolated clones by cDNA representational difference analysis between age matched normal and cd affected retinas. Among the clones isolated, we identified a cCRX transcript. However, linkage data already in hand (Acland et al., unpublished) which showed multiple recombinations to markers located in regions flanking cCRX [28] effectively and formally excludes cd from this entire interval on CFA1.

In addition, no abnormalities in the CRX sequence were found by comparing the exon sequences of normal dogs with those from dogs affected with rcd2 (collies), or early onset forms of PRA (American Staffordshire terrier, Tibetan terrier). This excludes coding and splicing mutations of CRX from involvement in the disease, but does not formally exclude the gene. However, by using markers close to CRX on CFA1 for linkage analysis, the candidate gene was excluded by finding multiple recombinants in the rcd2 and American Staffordshire terrier pedigrees. As multiple other genes and loci are associated with early onset rod and/or cone disease (RetNet), the exclusion of CRX from these four canine diseases is not surprising. Now that resources for linkage and physical mapping of disease traits are available for dogs [26,28,29], it will be possible to use the disease informative pedigrees to carry out genome wide scans. These will allow the use of comparative genomic approaches to identify the disease causing genes and mutations present in these important animal models.

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